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Crystallographic Evidence for Doxorubicin Binding to the Receptor-binding Site in *Clostridium botulinum* Neurotoxin B

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Beamline(s): X12C

Introduction: *Clostridium botulinum* and tetanus neurotoxins belong to the same class of neurotoxins and share significant sequence homology. They possess similar structural and functional domains and have a similar mechanism of toxicity. However, botulinum neurotoxins act at the neuromuscular junction (NMJ) causing flaccid paralysis, while tetanus toxin acts at the central nervous system (CNS) causing spastic paralysis. These neurotoxins are potential biowarfare agents and are also a significant public-health problem. There are no known antidotes available either for tetanus or for botulinum neurotoxins.

Clostridium botulinum toxins follow a four-step mechanism; they bind to the neuronal cells, are internalized into the vesicles and translocated into the cytosol, where they attack specific components of SNARE proteins to cleave them at specific peptide bonds causing inhibition of formation of SNARE complex and thereby blocking neurotransmitter release. *C*. neurotoxins comprise two chains, N-terminal light chain of 50 kDa and the C-terminal heavy chain of 100 kDa held together by a single disulfide bond. The heavy chain is responsible for binding, internalization and translocation, while the light chain is responsible for catalytic activity inside the cytosol. Neurotoxins bind to the neuronal cells *via* gangliosides and negatively-charged lipids on the surface of the cell (1). The catalytic domain is a zinc endopeptidase containing a HExxH zinc-binding motif (2).

Antagonists for these neurotoxins could act in three ways (3). Either they could be molecules which attach to the binding site thereby inhibiting binding of neurotoxins to gangliosides; or they may act before internalization to prevent internalization; or they could be inhibitors which would stop the catalytic action by blocking the active site or by chelating the active site zinc.

In an attempt to identify suitable small-molecule ligands that bind to the C-fragment of *C*. tetanus neurotoxin (TeNT), several small molecules were screened by computational chemistry and then tested with modeling, docking and electrospray ionization mass spectroscopy (ESI-MS) (4). Of the many compounds tested, doxorubicin, a DNA intercalator molecule, was identified to bind with a binding constant of 9.4 μM. Also, from the declustering potential used in ESI-MS, it was concluded that it binds in a hydrophobic pocket. It has been shown that it competes with gangliosides for binding. Here we present crystallographic evidence for doxorubicin binding to *C*. neurotoxins. We used BoNT/B in our studies since (1) a high-resolution structure was available; (2) all *C*. neurotoxins share significant sequence homology at the C-terminus and finally (3) they all possess similar structure (5-7)

Methods and Materials: BoNT/B crystals were obtained by the vapor diffusion method with PEG 4000 as precipitant in MES buffer (pH 6.0). The crystals belong to the space group $P2_1$ with cell dimensions a = 76.27, b = 122.93, c = 95.42 and b = 112.9 . The protein-doxorubicin complex crystals were prepared by soaking BoNT/B crystals in the mother liquid containing doxorubicin. The best soaking condition was obtained when crystals were soaked for 36 h in the mother liquid containing 50 mM doxorubicin.

Data were collected with this crystal at beam line X12C of National Synchrotron Light Source, Brookhaven National Laboratory with the use of CCD based detector (Brandies B1.2). The data extend to 2.5 resolution. The data collection and processing were done using MARMAD and HKL/Denzo (8). Since the crystals of BoNT/B are prone to non-isomorphism even among crystals from the same crystallization well the structure of the complex was determined by the molecular replacement method using the native structure as a model with AMoRe (9). The model was refined with CNS (10) until convergence. The sigma weighted difference Fourier density map was calculated and checked for the possible bound drug. With the available information about the sialyllactose binding site and the presence of a continuous residual density in the difference density map, the binding site of doxorubucin was identified and the ligand was modeled with the program 'O' (11). The final R and R-free are 0.22 and 0.28, respectively.

Results: The crystal structure of the BoNT/B-sialyllactose complex identified the potential receptor-binding site as the cleft between Trp1261 and His1240 (6). This binding pocket with a tryptophan exposed to solvent is commonly found in all the BoNTs and TeNT. The present study showed that Dox binds to the same site of sialic acid binding. His1240 and Trp1261 are on either side of Dox as with the sialyllactose. Even though the binding site was predicted by the previous study (4), the orientation of the molecule seems to be different from that proposed. Especially, the direction in which the amino group points is different; while it is pointing toward the interface of the two sub-domains of the C-fragment in the model proposed, it is pointing away from the interface in the crystal structure. This may provide an additional clue in designing molecules for inhibiting neurotoxin binding to the membranes.

Conclusions: The present study has defined the interactions between doxorubicin and the neurotoxin. Also, the difference in orientation of doxorubicin from that of the modeling studies underscores the importance of crystallographic study for understanding the interactions of drug molecules with toxins. Even though the affinity of doxorubicin to neurotoxins may not be strong, it certainly presents itself as a strong lead compound since a number of analogues of doxorubicin have already been synthesized and may present a better candidate. With the knowledge that doxorubicin competes with gangliosides to bind to the toxin and the mechanism being similar to the ganglioside binding, it would be a potential lead compound for drug design to treat botulism.

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